

## RESEARCH PAPER

# Early potassium channel blockade improves sepsis-induced organ damage and cardiovascular dysfunction

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## BACKGROUND AND PURPOSE

There is increasing evidence that potassium channels are involved in the cardiovascular dysfunction of sepsis. This evidence was obtained after the systemic inflammation, cardiovascular dysfunction and organ damage had developed. Here we have studied the consequences of early interference with potassium channels on development of sepsis.

## EXPERIMENTAL APPROACH

Sepsis was induced by caecal ligation and puncture (CLP) or sham surgery in Wistar rats. Four hours after surgery, animals received tetraethylammonium (TEA; a non-selective potassium channel blocker) or glibenclamide (a selective ATP-sensitive potassium channel blocker). Twenty-four hours after surgery, inflammatory, biochemical, haemodynamic parameters and survival were evaluated.

## KEY RESULTS

Sepsis significantly increased plasma NO<sub>x</sub> levels, expression of inducible nitric oxide synthase (NOS-2) protein in lung and thigh skeletal muscle, lung myeloperoxidase, urea, creatinine and lactate levels, TNF- $\alpha$  and IL-1 $\beta$ , hypotension and hyporesponsiveness to phenylephrine and hyperglycemia followed by hypoglycemia. TEA injected 4 h after surgery attenuated the increased NOS-2 expression, reduced plasma NO<sub>x</sub>, lung myeloperoxidase activity, levels of TNF- $\alpha$  and IL-1 $\beta$ , urea, creatinine and lactate levels, prevented development of hypotension and hyporesponsiveness to phenylephrine, the alterations in plasma glucose and reduced late mortality by 50%. Glibenclamide did not improve any of the measured parameters and increased mortality rate, probably due to worsening the hypoglycemic phase of sepsis.

## CONCLUSIONS AND IMPLICATIONS

Early blockade of TEA-sensitive (but not the ATP-sensitive subtype) potassium channels reduced organ damage and mortality in experimental sepsis. This beneficial effect seems to be, at least in part, due to reduction in NOS-2 expression.

## Abbreviations

1400W, N-(3-(aminomethyl)-benzyl)acetamidine; AG, aminoguanidine; CLP, caecal ligation and puncture; HR, heart rate; K<sub>ATP</sub> channel, ATP-sensitive potassium channel; K<sub>v</sub>, voltage-gated potassium channels; MAP, mean arterial pressure; NOS-2, inducible nitric oxide synthase; NO<sub>x</sub>, nitrite/nitrate; TEA, tetraethylammonium

## Introduction

Sepsis is an infection-induced systemic inflammatory syndrome, accompanied by hypotension due to systemic vasodi-

lation, and affects every major organ within the body, potentially leading to their failure (see O'Brien *et al.*, 2007).

Bacterial invasion releases inflammatory stimuli such as lipopolysaccharide (LPS), which evokes an overwhelming

spectrum of biological activities and pro-inflammatory proteins, among them the inducible nitric oxide (NO) synthase (NOS-2) isoform (see Knowles and Moncada, 1994; Assreuy, 2006). Activation of macrophages and cytokines by inflammation and the subsequent formation of reactive nitrogen species have currently been regarded as central pathogenic importance in sepsis.

Plasma membrane potential has important consequences for cell signalling; for instance, macrophage TNF- $\alpha$  release is highly dependent on the cell membrane potential (Haslberger *et al.*, 1992). Among other ion channels, potassium channels have an important role in macrophage activation being involved in IL-1 $\beta$  synthesis (Walev *et al.*, 1995), NOS-2 expression and NO production (Lowry *et al.*, 1998; Wu *et al.*, 1998) and TNF- $\alpha$  release (Blunck *et al.*, 2001; Seydel *et al.*, 2001). Several groups including our own have been studying the role of potassium channels as effectors of the vascular effects of NO during sepsis (Hall *et al.*, 1996; Taguchi *et al.*, 1996; Chen *et al.*, 1999; Silva-Santos *et al.*, 2002; see Fernandes and Assreuy, 2008). It is well known that NO opens potassium channels (Williams *et al.*, 1988; Fujino *et al.*, 1991) either via cGMP (Robertson *et al.*, 1993; Archer *et al.*, 1994) or directly (Bolotina *et al.*, 1994). There is increasing evidence from experimental studies that potassium channels are involved in the development of systemic vasodilatation and hyporesponsiveness to vasoconstrictors (Landry and Oliver, 1992; Vanelli *et al.*, 1995; Gardiner *et al.*, 1999; Lange *et al.*, 2006). However, these studies and others have examined the role of potassium channels in mediating NO effects after sepsis has been established. For example, injection of potassium channel blockers acutely improves the responsiveness to phenylephrine if given 24 h after sepsis induction (Sordi *et al.*, 2010). This beneficial effect however is evanescent and does not change the mortality or the general condition of the animals.

To better understand the role of potassium channels in sepsis, in this work, we employed two potassium channels blockers, tetraethylammonium (TEA) and glibenclamide, in the sepsis model of caecal ligation and puncture (CLP). The CLP model resembles ruptured appendicitis or perforated diverticulitis in humans, and it is pathophysiologically defined by two phases: a hyperdynamic phase (early) followed by a hypodynamic phase (late). The early phase is characterized by a hypermetabolic and hyperdynamic cardiovascular status, whereas the late phase sepsis is characterized by a hypometabolic and hypodynamic states (for review, see Buras *et al.*, 2005). Therefore, the purpose of the present work was to evaluate the consequences of early blockade of potassium channels on sepsis development, using an experimental model of sepsis in rats.

## Methods

### Animals

All animal care and experimental procedures were approved by the University Institutional Ethics Committee and are in accordance with CONCEA (National Council for the Control of Animal Experimentation) Guidelines. Female Wistar rats (weighing 200–250 g) were housed in a temperature- and

light-controlled room ( $23 \pm 2^\circ\text{C}$ ; 12 h light/dark cycle), with free access to water and food.

### CLP

Sepsis was induced with the CLP procedure, as previously described (Fernandes *et al.*, 2009). Briefly, rats were anaesthetized with ketamine and xylazine (90 and 15 mg·kg<sup>-1</sup> respectively, i.p.). Under aseptic conditions, a 2 cm midline laparotomy was performed to allow exposure of the caecum. The caecum was tightly ligated with a 3.0 silk suture at its base, below the ileocaecal valve, perforated 20 times with an 18 gauge needle, and a small amount of caecal content was squeezed through punctures. The caecum was replaced and the peritoneal wall and skin incisions were closed. Sham-operated rats underwent similar surgical procedures, but the caecum was not ligated or punctured. All animals received 10 mL·kg<sup>-1</sup> of sterile warm Dulbecco's phosphate-buffered saline (PBS; in mM, 137 NaCl, 2.7 KCl, 1.5 KH<sub>2</sub>PO<sub>4</sub> and 8.1 NaHPO<sub>4</sub>; pH 7.4) subcutaneously (s.c.).

### Mean arterial pressure (MAP) measurement

Under anaesthesia with ketamine and xylazine (as above, supplemented at 45 to 60 min intervals), heparinized PE-20 and PE-50 polyethylene catheters were inserted into the left femoral vein for drug injections and into the right carotid artery for recording of MAP respectively. Heart rate was derived from the blood pressure signal. To prevent clotting, a bolus dose of heparin (300 IU) was injected immediately after vein cannulation. Animals were allowed to breathe spontaneously via a tracheal cannula. Body temperature was monitored by a rectal thermometer and maintained at  $37 \pm 1^\circ\text{C}$  by means of a heating table. Blood pressure data were recorded with a catheter pressure transducer (Mikro-Tip®, Millar Instruments, Inc., Houston, TX, USA) coupled to a Powerlab 8/30 (AD Instruments Pty Ltd., Castle Hill, Australia). Results were expressed as mean  $\pm$  SEM of the peak changes in MAP (as mmHg) relative to baseline and recorded following administration of a given compound. At the end of the experiment, animals were killed by an overdose of chloral hydrate.

### Western blotting

Lung and thigh skeletal muscle tissues were homogenized in ice-cold buffer containing protease inhibitors (in mM: 50 HEPES, 1 MgCl<sub>2</sub>, 10 EDTA, and 1% Triton X-100, pH 6.4, containing 1  $\mu\text{g}\cdot\text{mL}^{-1}$  each of aprotinin, leupeptin, soy bean trypsin inhibitor and 1 mM phenylmethanesulphonyl fluoride). Protein samples (75  $\mu\text{g}\cdot\text{lane}^{-1}$ ) were subjected to denaturing gel electrophoresis (SDS-PAGE, 8% gel). After electrophoresis, proteins were electro-transferred to PVDF membranes (0.8 mA/cm<sup>2</sup>; 15 V in Tris-glycine buffer composed by 48 mM Tris-HCl/39 mM glycine/10% methanol). The membrane was incubated for 1 h at room temperature with T-PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 10.8 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O and 0.05% Tween-20, pH 7.4) containing 5% skimmed milk. Membranes were then incubated with rabbit polyclonal anti-NOS-2 antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-actin antibody (1:1000; Santa Cruz Biotechnology) overnight at 4°C. Membranes were washed and incubated with biotinylated secondary antibodies (1:1000; Amersham Biosciences, Buck-

inghamshire, UK) and then with streptavidin horseradish peroxidase conjugate (1:1000; Amersham Biosciences). Immunocomplexes were visualized by chemiluminescence, and band intensity was quantified by densitometry using Scion Image software (Scion Co., Frederick, MD, USA).

### *NO<sub>x</sub> (nitrite + nitrate) assay*

Briefly, zinc sulphate-deproteinized plasma samples were subjected to nitrate conversion. Nitrate was converted to nitrite using *Escherichia coli* nitrate reductase for 3 h at 37°C. Samples were centrifuged to remove bacteria, and 100 µL of each sample was mixed with Griess reagent (1% sulphanilamide in 10% phosphoric acid/0.1% naphthylethylenediamine in Milli-Q water) in a 96-well plate and read at 540 nm in a plate reader. Standard curves of nitrite and nitrate (0–150 µM) were run simultaneously. As under these conditions nitrate conversion was always greater than 90%, no corrections were made. Values are expressed as µM NO<sub>x</sub> (nitrate + nitrite).

### *Myeloperoxidase (MPO) assay*

Lungs were homogenized in hexadecyltrimethylammonium bromide buffer (HTAB; 0.5% in 80 mM sodium phosphate buffer, pH 5.4). Supernatant was assayed for MPO activity using a published method (Bradley *et al.*, 1982). Total protein was determined according the Bradford method. Myeloperoxidase activity was expressed as optical density at 450 nm per mg of protein.

### *Glucose, urea, creatinine and lactate levels*

Tail blood glucose was measured electrochemically by using Tracase Smart System (Home Diagnostics, Inc., Fort Lauderdale, FL, USA). Plasma levels of urea nitrogen, creatinine and lactate were measured by using commercially available clinical assay kits (Gold Analisa Diagnostica and BioClin, Belo Horizonte, MG, Brazil).

### *Leukocyte and blood bacteria counts*

Total leukocyte numbers were determined by haemocytometer cell counter and was reported as 10<sup>3</sup> cells mm<sup>-3</sup>. Blood was collected 24 h after CLP or sham surgery under sterile conditions. Serial 10-fold dilutions of blood were prepared in sterile water, and 10 µL of each dilution was plated on Mueller-Hinton agar dishes and incubated at 37°C; colony-forming units (CFU) were analysed after 24 h, and the results were expressed as CFU mL<sup>-1</sup> of blood.

### *Determination of cytokine levels*

TNF-α and IL-1β in serum were measured by commercially available enzyme-linked immunosorbent assay kits (Pepro-Tech Inc., Rocky Hill, NJ, USA).

## Experimental design

### *Survival*

Rats were sham-operated or submitted to CLP as described above and randomized to receive TEA (8 or 16 mg·kg<sup>-1</sup>; s.c.), glibenclamide (1 or 20 mg·kg<sup>-1</sup>; s.c.) or vehicle [PBS or dim-

ethyl sulphoxide (DMSO) respectively] 4 h after CLP procedure. Groups (TEA or glibenclamide) were studied on different days, and each group was compared with its control group, because they had received different vehicles (PBS or DMSO). The injected volume of DMSO was never higher than 0.5 mL·kg<sup>-1</sup>. In order to evaluate the effect of a partial or a total inducible NOS-2 inhibition on survival rate, 12 h after CLP surgery, a group of animals received aminoguanidine (10 or 200 mg·kg<sup>-1</sup>; s.c.) or 1400W [N-(3-(aminomethyl)-benzyl)acetamide; 0.3 mg·kg<sup>-1</sup>; s.c.] or vehicle (PBS). Survival was recorded every 12 h over a 3 day period. Doses of potassium channel blockers and NOS-2 inhibitors were chosen based on our own experience (Silva-Santos and Assreuy, 1999; Silva-Santos *et al.*, 2002; Sordi *et al.*, 2010) and from others (Sorrentino *et al.*, 1999; Benjamim *et al.*, 2000).

### *Vascular reactivity and tissue collection*

Rats were sham-operated or submitted to CLP as described above and randomized to receive TEA (8 mg·kg<sup>-1</sup>; s.c.), glibenclamide (1 mg·kg<sup>-1</sup>; s.c.) or vehicle (PBS or DMSO) 4 h after CLP surgery. Twenty hours later, animals were prepared for MAP recording, and a randomized dose-response curve to phenylephrine (3, 10 and 30 nmol·kg<sup>-1</sup>) was obtained. Each dose of the vasoconstrictor was given only when the pressor effect of the preceding one had fully subsided. Basal MAP and heart rate (HR) were also recorded. Immediately after the experiment, blood samples were obtained for measurement of plasma urea, creatinine and NO<sub>x</sub> levels and lung and thigh skeletal muscle tissues for NOS-2 immunoelectrophoresis. For cytokine measurement, blood samples were collected, and serum was obtained for the assays. An additional group of animals was included where animals received NOS-2 inhibitors (1400W 0.3 mg·kg<sup>-1</sup> or aminoguanidine 10 or 200 mg·kg<sup>-1</sup>) 12 h after surgery, and blood samples were obtained for plasma NO<sub>x</sub> levels measurement.

### *Blood glucose measurement*

Rats were sham- or CLP-operated and randomized to receive TEA (8 mg·kg<sup>-1</sup>; s.c.), glibenclamide (1 mg·kg<sup>-1</sup>; s.c.) or vehicle (PBS or DMSO) 4 h after the surgery. Six, 12 and 24 h after CLP procedure, 1 µL of blood was obtained from the tail tip, and blood glucose levels were measured as described above.

### *Statistical analysis*

Data are expressed as mean ± SEM of *n* animals. Statistical significance was analysed by two-way analysis of variance (ANOVA) followed by the appropriate *post hoc* test as indicated in the figure legends. Differences in the survival were determined with log rank test. A *P*-value of less than 0.05 was considered significant. Two-way ANOVA analyses were performed using the Statistica® software package (StatSoft Inc., Tulsa, OK, USA), and the other tests were performed using GraphPad Prism software (San Diego, CA, USA).

### *Materials*

TEA chloride, aminoguanidine, 1400W, glibenclamide and phenylephrine chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Heparin was a kind gift of Cristália Laboratories (São Paulo, SP, Brazil). All drug and receptor nomenclature follows Alexander *et al.*, 2009.

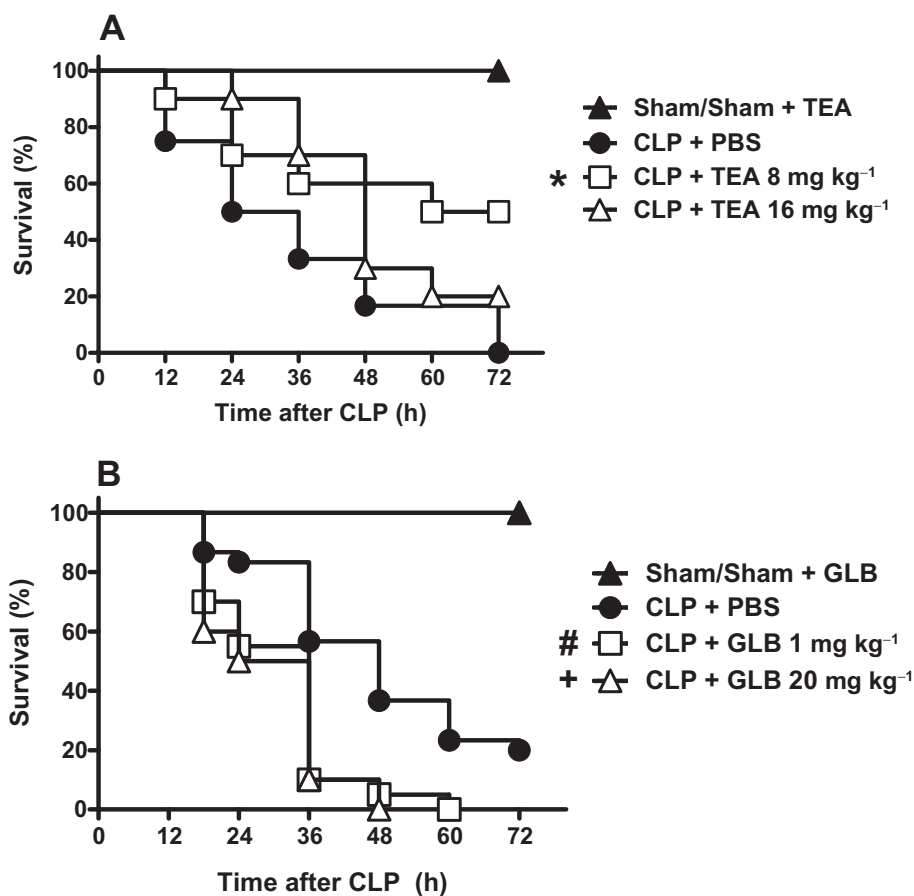
## Results

### Effects of potassium channel blockers on survival rate

Sepsis induced a progressive mortality in animals submitted to CLP surgery (Figure 1). TEA treatment ( $8 \text{ mg}\cdot\text{kg}^{-1}$ ) given 4 h after CLP surgery protected half of animals from death. Curiously, a higher dose of TEA ( $16 \text{ mg}\cdot\text{kg}^{-1}$ ) did not change the mortality when compared with CLP control group (Figure 1A). On the other hand, glibenclamide treatment in both doses increased CLP mortality (Figure 1B). No mortality was observed in sham, sham + TEA or sham + glibenclamide groups (Figure 1 A and B). Based on this result, the subsequent experiments were performed with TEA  $8 \text{ mg}\cdot\text{kg}^{-1}$  and glibenclamide  $1 \text{ mg}\cdot\text{kg}^{-1}$ .

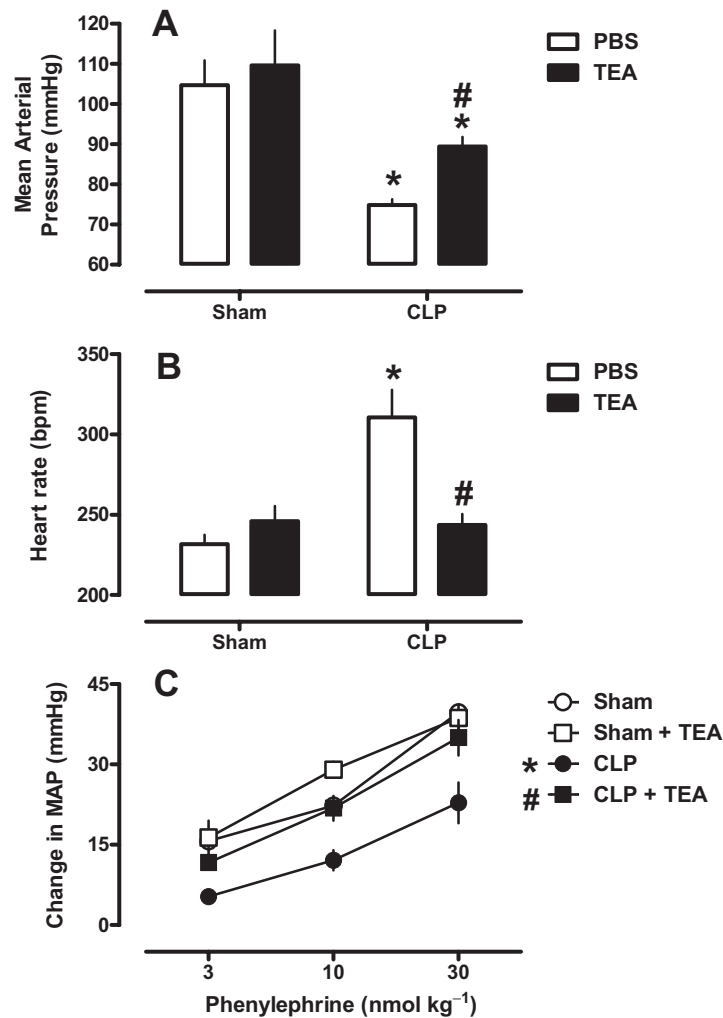
### Effects of potassium channel blockers on MAP, HR and vasoconstrictor response

Sham animals exhibited a MAP of  $105 \pm 6 \text{ mmHg}$  and a HR of  $232 \pm 6 \text{ bpm}$ . Twenty-four hours after CLP procedure, MAP had decreased (Figure 2A), and heart rate was increased (Figure 2B), both by 30%. In addition, there was a substantial reduction (about 50%) in phenylephrine response (Figure 2C). The early injection (4 h after CLP surgery) of only one dose of TEA was enough to completely restore the HR and phenylephrine response to normal values. Early TEA injection, however, only partially blocked the development of hypotension. TEA did not affect MAP, HR or phenylephrine response in sham-operated animals (Figure 2). On the other hand, the early injection of glibenclamide did not change the sepsis-induced cardiovascular dysfunction (Figure 3, Panel A, B and C).



**Figure 1**

Effect of potassium channels blockers on survival of septic rats. A. Effect of tetraethylammonium (TEA). Rats were sham-operated or submitted to CLP procedure. CLP animals were treated subcutaneously with PBS, TEA  $8 \text{ mg}\cdot\text{kg}^{-1}$  or TEA  $16 \text{ mg}\cdot\text{kg}^{-1}$  4 h after the surgery. Sham animals received PBS or TEA and, because there was no difference between these groups, their values are presented as one group. B. Effect of glibenclamide (GLB). CLP animals were treated subcutaneously with DMSO or GLB  $1 \text{ mg}\cdot\text{kg}^{-1}$  or  $20 \text{ mg}\cdot\text{kg}^{-1}$  4 h after surgery. Sham-operated animals that received DMSO or glibenclamide were grouped together because there was no difference between them. Statistical analysis was performed using log-rank test. \* $P = 0.01$ , # $P = 0.0004$ , + $P = 0.001$  significantly different from CLP+PBS rats,  $n = 20$  per group. Results were expressed as percent survival and are representative of two different experiments. The significance symbols beside the survival curve means that the curve was statistically different from the reference survival (CLP+PBS).



**Figure 2**

Effects of tetraethylammonium (TEA) on haemodynamic parameters of rats after CLP. Rats were injected with TEA ( $8 \text{ mg}\cdot\text{kg}^{-1}$ , s.c.) 4 h after CLP or sham surgery. Twenty-four hours after surgery, mean arterial pressure (A), heart rate (B) and phenylephrine dose–response curves (C) were evaluated. Each bar represents the mean of six to eight animals, and vertical lines are the SEM. Statistical analyses were performed using two-way ANOVA followed by Newman–Keuls *post hoc* test. \* $P < 0.05$  significantly different from sham-operated group and # $P < 0.05$  significantly different from CLP group.

### Effects of potassium channel blockers on organ damage and inflammatory parameters

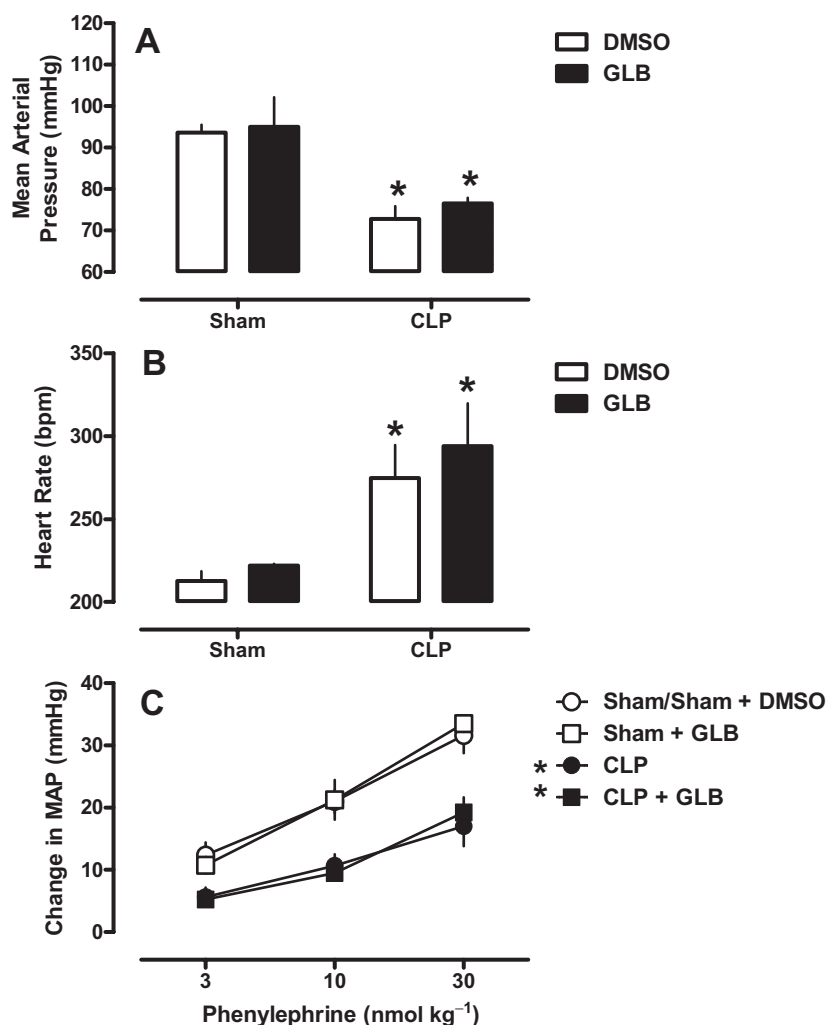
Sepsis induced renal damage and tissue hypoperfusion as demonstrated by increased plasma levels of urea, creatinine and lactate. CLP animals developed leucopenia and increased myeloperoxidase activity in the lung (Tables 1 and 2). Levels of the cytokines TNF- $\alpha$  and IL-1 $\beta$  were increased in serum from rats after CLP surgery (Figure 4). Early TEA treatment reduced the magnitude of all these changes after CLP (Table 1 and Figure 4, Panels A and C), except the leucopenia. On the other hand, glibenclamide treatment (Table 2) either failed to improve or exacerbated these changes. Neither the potassium channels blockers nor their vehicles changed these variables in sham-operated animals.

### Effects of potassium channel blockers on plasma NO<sub>x</sub> levels

NO<sub>x</sub> levels in plasma were increased in animals after CLP (Figure 5). Treatment of CLP rats with TEA significantly reduced plasma NO<sub>x</sub> levels (Figure 5A), whereas GLB was without effect (Figure 5B). None of the blockers or their vehicles altered NO<sub>x</sub> levels in sham-operated animals (Figure 5 A and B).

### Effects of TEA on lung and thigh skeletal muscle NOS-2 expression

NOS-2 protein in extracts of lung or thigh skeletal muscle was increased by CLP, when measured 24 h later. TEA injected 4 h



**Figure 3**

Effects of glibenclamide (GLB) on haemodynamic parameters of rats after CLP. Rats were injected with glibenclamide (1 mg·kg<sup>-1</sup>, s.c.) or vehicle (DMSO; 100 µL) 4 h after CLP or sham surgery. Twenty-four hours after surgery, mean arterial pressure (A), heart rate (B) and phenylephrine dose–response curves (C) were evaluated. Each bar represents the mean of six to eight animals, and vertical lines are the SEM. Statistical analysis was performed using two-way ANOVA followed by Newman–Keuls *post hoc* test. \**P* < 0.05 significantly different from corresponding sham group.

**Table 1**

Effects of the early injection of tetraethylammonium (TEA) on blood biochemistry and inflammatory markers in rats after CLP or sham surgery

	Experimental groups			
	Sham PBS	TEA	CLP PBS	TEA
Urea	217 ± 15	222 ± 27	390 ± 51*	247 ± 23 <sup>#</sup>
Creatinine	2.0 ± 0.1	2.4 ± 0.4	4.4 ± 0.4*	3.1 ± 0.2* <sup>#</sup>
Lactate	91 ± 32	75 ± 14	221 ± 26*	129 ± 16 <sup>#</sup>
Leukocytes	7.7 ± 0.68	8.0 ± 0.45	4.6 ± 0.49*	4.9 ± 0.65*
MPO activity	63 ± 11.6	93 ± 4.7	202 ± 24.6*	143 ± 12.1* <sup>#</sup>

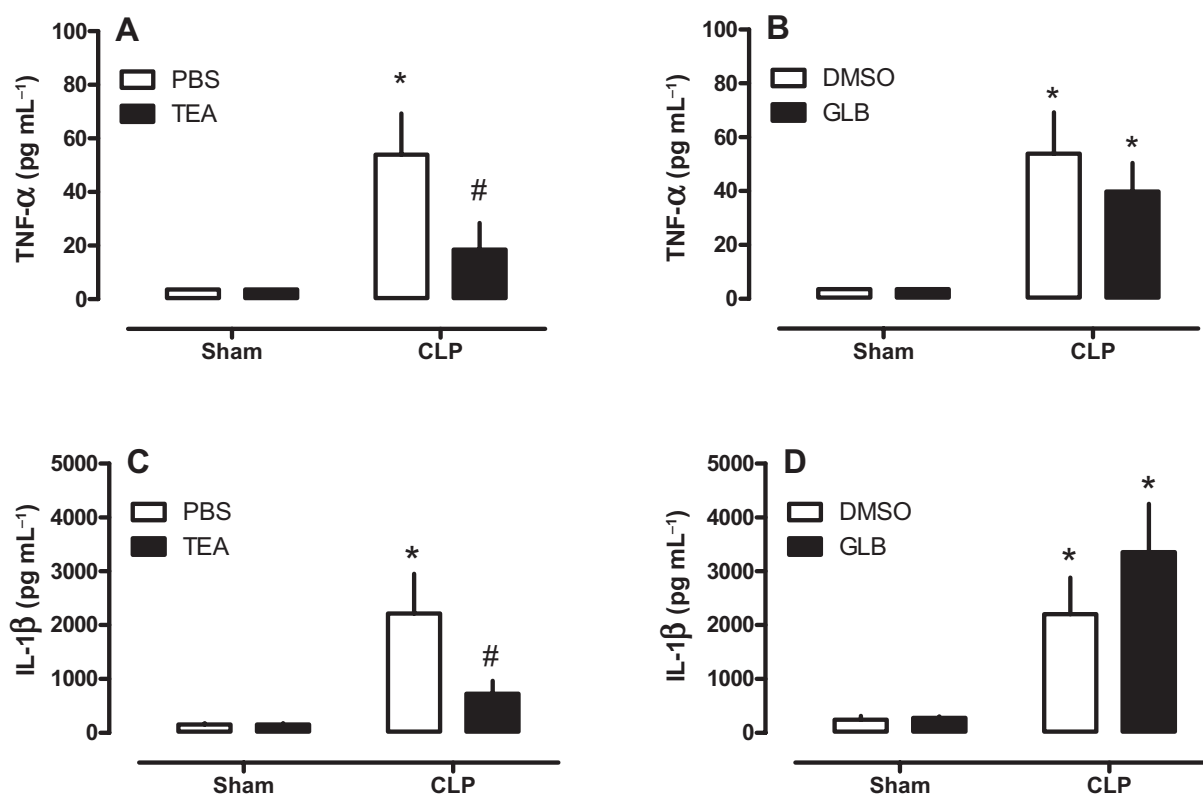
Rats were injected with TEA (8 mg·kg<sup>-1</sup>, s.c.) or vehicle (PBS) 4 h after CLP or sham surgery and the variables measured 20 h later. Urea, creatinine and lactate values are expressed as mg L<sup>-1</sup>; lung myeloperoxidase (MPO) activity is expressed as optical density units (mg protein)<sup>-1</sup>; total leukocyte numbers are expressed as 10<sup>3</sup> cells mm<sup>-3</sup>. Data are expressed as mean ± SEM. Statistical analyses were performed using two-way ANOVA followed by Newman–Keuls *post hoc* test. \**P* < 0.05 significantly different from sham animals; <sup>#</sup>*P* < 0.05 significantly different from CLP animals.

**Table 2**

Effects of the early injection of glibenclamide (GLB) on blood biochemistry and inflammatory markers in rats after CLP or sham surgery

	Experimental Group			
	Sham DMSO	GLB	CLP DMSO	GLB
Urea	284 ± 17	312 ± 31	615 ± 151*	429 ± 66*
Creatinine	1.7 ± 0.5	1.9 ± 0.4	3.9 ± 0.5*	3.4 ± 0.4*
Lactate	245 ± 17	226 ± 24	357 ± 28*	340 ± 11*
Leukocytes	7.7 ± 0.66	7.1 ± 0.24	4.7 ± 0.11*	5.1 ± 0.47*
MPO activity	118 ± 23.1	90 ± 10.1	294 ± 63.8*	306 ± 74.5*

Rats were injected with glibenclamide (1 mg·kg<sup>-1</sup>, s.c.) or vehicle (DMSO) 4 h after CLP or sham surgery and the variable measured 20 h later. Other details are as in Table 1.

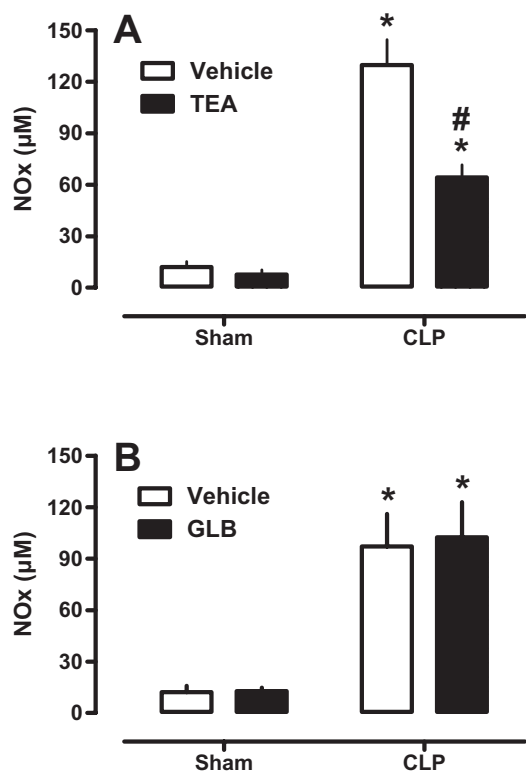
**Figure 4**

Effects of potassium channel blockers on serum cytokine levels. Rats were injected with vehicle (PBS or DMSO), tetraethylammonium (TEA; A and C; 8 mg·kg<sup>-1</sup>, s.c.) or glibenclamide (GLB; B and D; 1 mg·kg<sup>-1</sup>, s.c.) 4 h after CLP or sham surgery. Twenty hours later, blood samples were collected and assayed for cytokine levels; TNF-α (A and B); IL-1 β (C and D). Each bar represents the mean of 8–10 animals, and vertical lines are SEM. Statistical analyses were performed using two-way ANOVA followed by Newman–Keuls *post hoc* test. \**P* < 0.05 significantly different from corresponding sham group and #*P* < 0.05 significantly different from corresponding CLP rats.

after CLP surgery reduced these levels in lung (Figure 6 A and B) and thigh skeletal muscle (Figure 6 A and C). Levels of β-actin in these tissues were not altered by CLP surgery or TEA treatment (Figure 6 A). Because glibenclamide did not alter NO<sub>x</sub> levels (Figure 5 B), NOS-2 expression was not studied in this group.

### Effects of potassium channel blockers on blood bacterial counts in sepsis

The bacterial load in blood samples obtained from CLP rats was, as expected, significantly higher than in the sham group (Figure 7). Early treatment with potassium channel blockers



**Figure 5**

Effects of potassium channel blockers on NO<sub>x</sub> plasma levels. Rats were injected with vehicle (PBS or DMSO), tetraethylammonium (TEA; A; 8 mg·kg<sup>-1</sup>; s.c.) or glibenclamide (GLB; B; 1 mg·kg<sup>-1</sup>; s.c.) 4 h after CLP or sham surgery. Twenty hours later, blood samples were collected and assayed for NO<sub>x</sub> levels. Each bar represents the mean of 8–10 animals, and vertical lines are SEM. Statistical analyses were performed using two-way ANOVA followed by Newman–Keuls *post hoc* test. \**P* < 0.05 significantly different from corresponding sham group and #*P* < 0.05 significantly different from corresponding CLP rats.

did not change bacterial counts in the blood of sham or CLP animals.

### Effects of potassium channel blockers on blood glucose levels

CLP surgery induced biphasic changes in blood glucose. Six hours after CLP, animals presented with hyperglycemia, and, from 12 h onwards, a progressive hypoglycemia developed (Figure 8 A). Treatment of CLP rats with TEA prevented both sepsis-induced hypoglycemia and hyperglycemia (Figure 8 C). In both sham and sham + TEA groups, blood glucose levels were not significantly changed during the experimental period (Figure 8 B). On the other hand, glibenclamide reduced blood glucose levels in sham-operated animals (Figure 8 D) and worsened the hypoglycemic phase in CLP animals (Figure 8 E).

### Effects of inducible NOS-2 inhibition on NO<sub>x</sub> levels and survival

Taking into account that the partial reduction in NOS-2 levels had several beneficial effects, we wondered if a partial reduc-

tion in NOS-2 activity (assessed by plasma NO<sub>x</sub>) would have a similar outcome. The effects of NOS-2 inhibition on survival rate were assessed using a lower and a higher dose of aminoguanidine (10 and 200 mg·kg<sup>-1</sup>; i.p.) or a low dose of 1400W (0.3 mg·kg<sup>-1</sup>; i.p.), given to the animals 12 h after surgery. The lower dose of aminoguanidine improved the survival, whereas the high dose did not (Figure 9A). The low dose of 1400W, considered a selective NOS-2 inhibitor, also improved survival (Figure 9B). NO<sub>x</sub> levels in animals with sepsis and treated with the lower dose of both inhibitors were partially reduced (Figure 10 A and B, respectively), whereas those after treatment with the higher dose of aminoguanidine were similar to levels found in control animals (Figure 10 A).

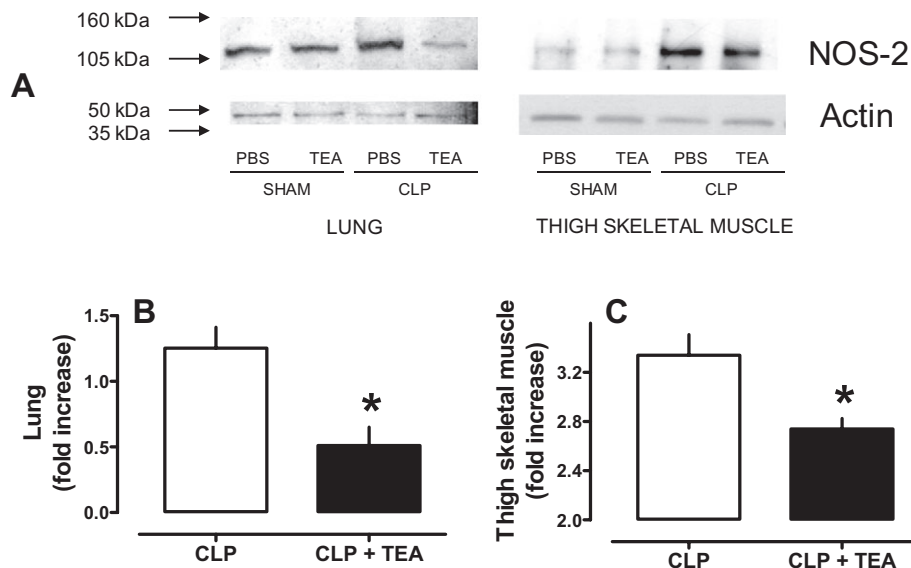
## Discussion and conclusions

The main finding of the present report is that the early blockade of potassium channels with TEA dramatically improves cardiovascular and inflammatory parameters and decreases the mortality rate of septic rats. On the other hand, the selective blockade of ATP-sensitive potassium (K<sub>ATP</sub>) channel by glibenclamide not only did not provide any improvement but actually increased mortality.

This latter finding was somewhat surprising because there are several reports showing beneficial effects of K<sub>ATP</sub> channel inhibitors in experimental sepsis and endotoxemia. For instance, intravenous infusion of glibenclamide reversed endotoxin-induced systemic hypotension in dogs (Landry and Oliver, 1992), pigs (Vanelli *et al.*, 1995) and rats (Gardiner *et al.*, 1999). Two recent studies investigated the effect of K<sub>ATP</sub> channel blockade in patients with septic shock (Warrillow *et al.*, 2006; Morelli *et al.*, 2007) and, in contrast to the animal studies, glibenclamide failed to improve haemodynamics or to reduce noradrenaline requirements in both studies. Although these studies have been questioned (Oliver and Landry, 2006), they would be compatible with our results. Moreover, recent work shows that improvement in sepsis survival by hydrogen sulphide was mediated by K<sub>ATP</sub> channel activation and glibenclamide treatment prevented these beneficial effects (Spiller *et al.*, 2010). Our results point to the activation, and not the blockade, of K<sub>ATP</sub> channels as being beneficial in sepsis.

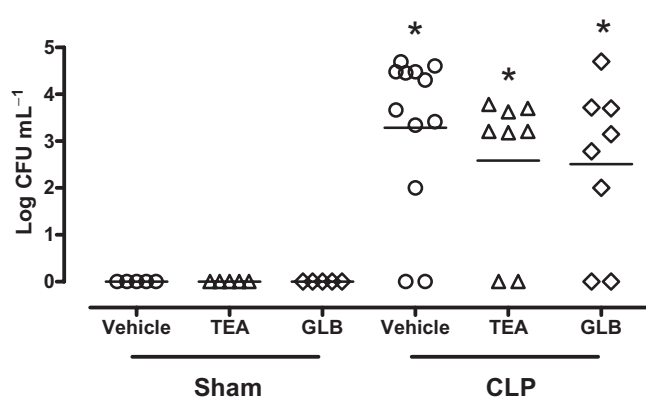
In striking contrast, the early treatment with only one injection of TEA improved the inflammatory and dysfunctional haemodynamic parameters of septic animals. TEA treatment early in sepsis reduced the late consequences of the systemic inflammation in septic rats, as shown by the reduced myeloperoxidase activity and decrease in NO<sub>x</sub>, TNF-α and IL-1β levels. These data corroborate previous work showing that potassium channels are involved in transmembrane signal transduction in macrophages and that blockers of these channels reduced cytokine production in these cells (Blunck *et al.*, 2001; Papavlassopoulos *et al.*, 2006). Animals with sepsis also exhibited a decrease in blood leukocytes. The leukocyte number drops during sepsis because these cells, mainly neutrophils, transmigrate to the infectious focus and to other organs such as the lung (Alves-Filho *et al.*, 2008), which in turn contributes to the multiple organ dysfunction of sepsis (Souto *et al.*, 2011). This is confirmed by the increase in lung MPO activity in rats after CLP, reflecting a systemic inflammation that the treatment with TEA was able to reduce. The reduced





**Figure 6**

Effect of tetraethylammonium (TEA) on inducible nitric oxide synthase (NOS-2) expression. A. Representative immunoelectrophoresis of NOS-2 and  $\beta$ -actin in extracts of lung and thigh skeletal muscle from rats after CLP and treated with TEA ( $8 \text{ mg}\cdot\text{kg}^{-1}$ ; s.c.) 4 h after surgery. Tissues were harvested 24 h after CLP, homogenized and centrifuged. The same amount of protein ( $75 \mu\text{g}$ ) was loaded in each well, and a representative experiment is shown. Band intensity of lung (B) and thigh skeletal muscle (C) NOS-2 were quantified by densitometry, and all data have been normalized to the levels of sham-operated animals. Each bar represents the mean of three to four independent experiments, and vertical lines are SEM. Statistical analysis was performed using two-way ANOVA followed by Newman-Keuls *post hoc* test.  $*P < 0.05$  significantly different from corresponding CLP group.

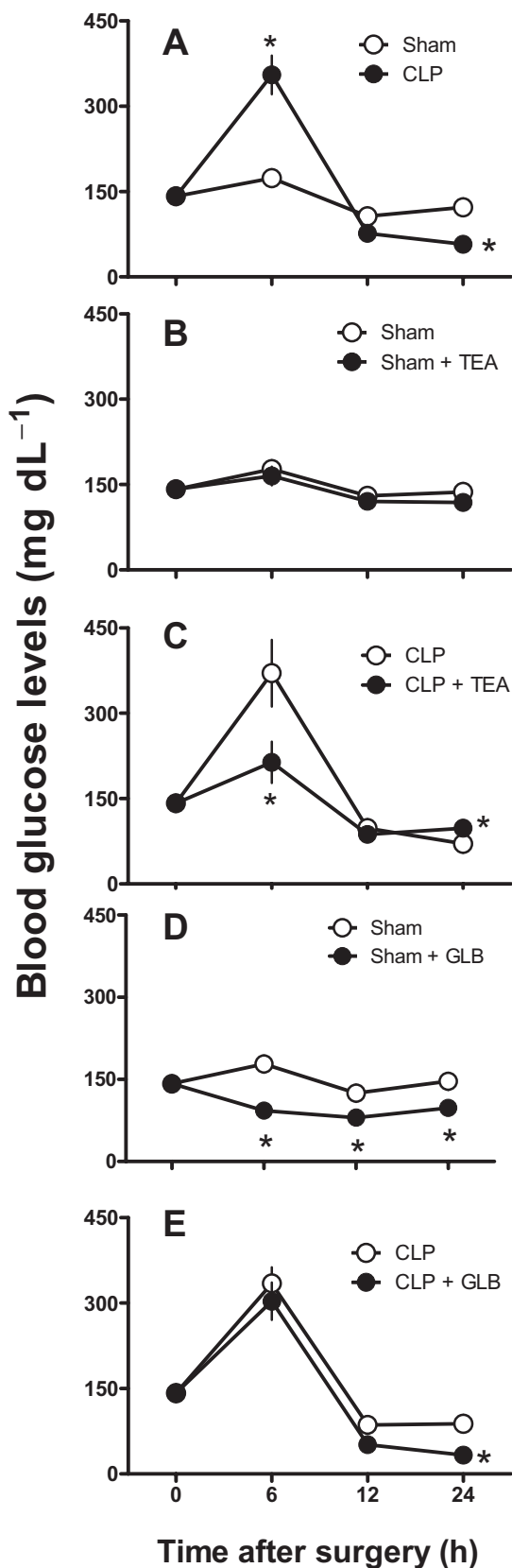


**Figure 7**

Effects of potassium channel blockers on blood bacterial load. Animals were treated 4 h after sham or CLP surgery with vehicle (PBS or DMSO), tetraethylammonium (TEA;  $8 \text{ mg}\cdot\text{kg}^{-1}$ ; s.c.) or glibenclamide (GLB;  $1 \text{ mg}\cdot\text{kg}^{-1}$ ; s.c.). Peripheral blood was collected 24 h after CLP, and serial dilutions were placed on Mueller-Hinton agar plates. Colony-forming units (CFU) in blood were determined 24 h after plating and were expressed as log CFU  $\text{mL}^{-1}$  blood. Individual animals are represented by individual symbols. Sham and CLP control animals received PBS or DMSO and, because there was no difference between these groups, their values were presented as one group (vehicle). Statistical analyses were performed using two-way ANOVA followed by Newman-Keuls *post hoc* test.  $*P < 0.05$  significantly different from corresponding sham group.

systemic inflammatory response, in turn, diminished the development and/or maintenance of organ dysfunction and hence would contribute to the increased survival.

It is important to note that the reduction in  $\text{NO}_x$  levels was *partial* and probably due to a similarly partial reduction in NOS-2 expression. Although certainly not the only relevant mechanism, NO/NOS-2 reduction can be regarded as an important contribution to the beneficial effects of TEA. Non-selective NOS inhibition has harmful consequences to the host (Lopez *et al.*, 2004). However, our results suggest that a *partial* reduction in NOS-2 expression may be advantageous in sepsis because it seemed to reduce the unwanted cardiovascular and perfusional consequences of increased NO synthesis, while preserving other important NO actions, such as host defense. Interestingly enough, the partial reduction of NOS-2 expression, although not affecting the bacteremia, had important consequences for the cardiovascular system. TEA was able to prevent hypotension and vascular hyporesponsiveness to phenylephrine and reduced hypoxia and tissue damage as suggested by the decrease in lactate, urea and creatinine levels. Following the same line of reasoning, if TEA had totally inhibited NOS-2 expression, we would expect that the ability of the host to deal with the infection would be compromised. However, our results show that the bacterial burden was not changed by the early injection of TEA and the reduced NOS-2 expression, suggesting the ability to deal with the infection was unaffected, even with reduced cytokine levels. Although NO and cytokines are important to host defense, others mechanisms such as the complement system and reactive oxygen species are also important and may compensate for the reduction in NO and cytokine production.



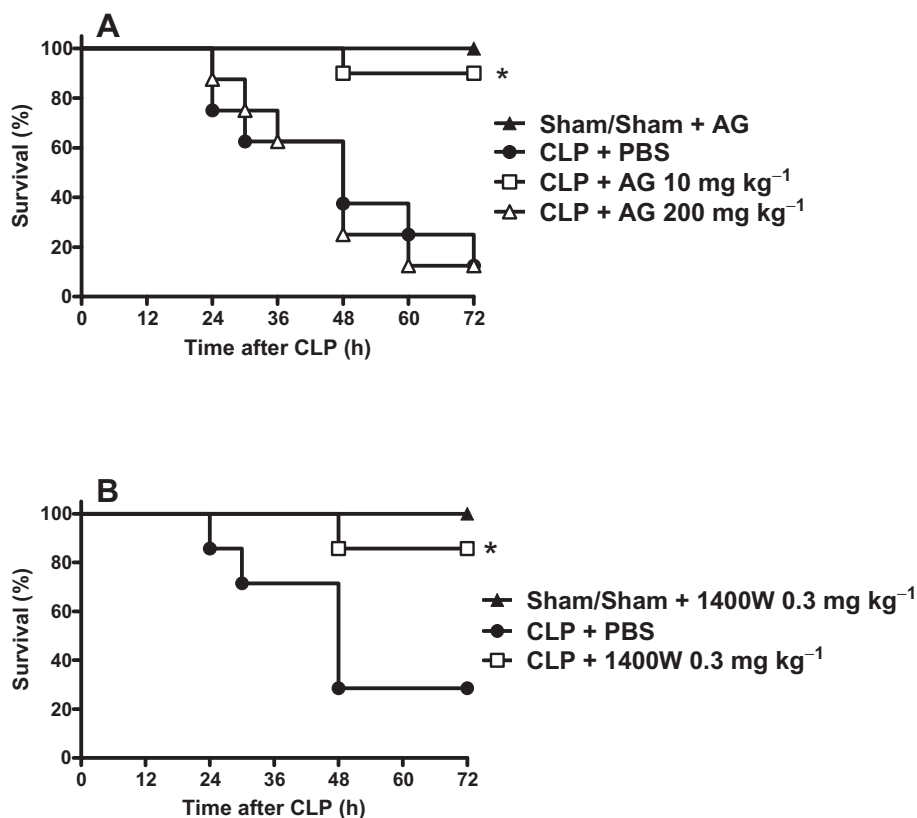
**Figure 8**

Effect of potassium channel blockade on blood glucose level changes induced by CLP. Six, 12 and 24 h after CLP, blood glucose levels were measured electrochemically in blood withdrawn from the tail tip. A. Blood glucose level in sham and CLP animals. B and D. Sham animals were randomized and 4 h after surgery treated with tetraethylammonium (TEA) 8 mg·kg<sup>-1</sup> (B), glibenclamide (GLB) 1 mg·kg<sup>-1</sup> (D); or vehicle. C and E. CLP animals were randomized and 4 h after surgery were treated with TEA 8 mg·kg<sup>-1</sup> (C), GLB 1 mg·kg<sup>-1</sup> (E) or vehicle. Data were expressed as mean ± SEM from four to six animals. Statistical analysis was performed using two-way ANOVA followed by Newman-Keuls *post hoc* test. \**P* < 0.05 significantly different from corresponding sham-operated group and #*P* < 0.05 significantly different from corresponding CLP control group.

The consequences of the partial reduction in NO production induced by TEA suggested that this would be advantageous to animals with sepsis. As we found, the increase in survival after low doses of aminoguanidine and 1400W was so clear that this strategy is worth exploring in clinical studies. Another interesting aspect of our results is that the higher dose of aminoguanidine probably lost selectivity for the NOS-2 isoform, corroborating the results of a study in humans, indicating that non-selective NOS inhibition was harmful to the host (Lopez *et al.*, 2004).

In contrast to our data, previous studies of TEA in sepsis have shown no improvement in blood pressure or mortality (Clayton *et al.*, 2005; Cauwels and Brouckaert, 2008). The most likely explanation for the discrepancy between our data and these two studies lies with the animal model used and the dose of TEA. TEA is regarded as a non-selective inhibitor of potassium channels; at low doses, TEA inhibits preferentially calcium-activated potassium channels, whereas at higher doses, it inhibits most, if not all, potassium channel subtypes (Nelson and Quayle, 1995). In our study, only the lower dose of TEA was effective in reducing the mortality, whereas the higher dose had no effect on survival. Maybe the higher dose of TEA inhibited other potassium channel subtypes (including the ATP-sensitive subtype) that somehow counteracted the beneficial effects of the lower dose. TEA reversed the attenuated vasoconstrictor response to noradrenaline during experimental human endotoxemia mediated by NO-induced opening of calcium-activated potassium channels (Pickkers *et al.*, 2006), suggesting that the beneficial effects of a lower dose of TEA shown here might be due to inhibition of calcium-activated potassium channels. This suggestion was strengthened by unpublished experiments from our group with the K<sub>v</sub> channel blocker 4-aminopyridine (10 and 100 µg·kg<sup>-1</sup>; s.c.; 4 h after surgery). Blockade of this potassium channel subtype did not induce any effects, either protective or harmful, in animals with sepsis.

As in human sepsis, CLP in rodents results in an early hyperdynamic and hypermetabolic phase followed by a later hypodynamic and hypometabolic phase (Siegel, 1981; Maitra *et al.*, 2000). As can be seen from our results, experimental sepsis in rats reproduced the metabolic biphasic pattern. Hyperglycemia is a complication that contributes to morbidity and mortality of septic shock (Buras *et al.*, 2005). It is well established that hyperglycemia has toxic effects besides increasing susceptibility to infection and the severity of hyperglycemia has been repeatedly associated with adverse



**Figure 9**

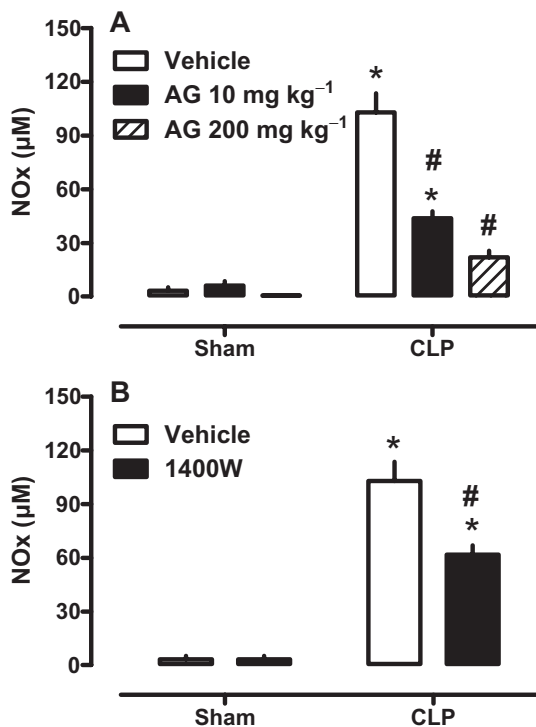
Effect of NOS-2 inhibitors on the survival of rats with sepsis. A. Aminoguanidine (AG). Rats were sham-operated or submitted to CLP procedure. CLP animals were treated subcutaneously with PBS, AG 10 mg·kg<sup>-1</sup> or 200 mg·kg<sup>-1</sup>, 12 h after the surgery. Sham animals received PBS or AG, and because there was no difference between these groups, their values are presented as one group. Statistical analysis was performed using log-rank test. \* $P < 0.0013$  significantly different from CLP+PBS rats,  $n = 10$  per group. B. Effects of 1400W. CLP animals were treated subcutaneously with PBS or 1400W 0.3 mg·kg<sup>-1</sup> 12 h after surgery. Sham-operated animals that received PBS or 1400W were grouped together because there was no difference between them. Statistical analysis was performed using log-rank test. \* $P < 0.007$  significantly different from CLP+PBS rats,  $n = 10$  per group. Results were expressed as percent survival and are representative of two different experiments.

outcomes of sepsis (Van den Berghe, 2003; Vanhorebbek *et al.*, 2007). In our hands, TEA treatment prevented the biphasic changes in blood glucose levels observed in animals with sepsis. The lack of effect on sham-operated rats of glycaemia is further evidence that the protective effect of TEA was not due to  $K_{ATP}$  channel blockade. As expected, glibenclamide decreased blood glucose levels of sham animals, but it did not prevent sepsis-induced hyperglycemia and in fact worsened the hypoglycemic phase of septic shock. This fact probably contributed to the increased mortality in rats treated with glibenclamide. To assess whether glibenclamide-induced hypoglycemia could be masking a putative protective effect on mortality, glucose was supplemented to septic animals treated with glibenclamide. Although infusion of glucose O'Brien *et al.* (2009) was not feasible in our protocols, injection of glucose every 8 h in rats after CLP and treated with glibenclamide provided blood glucose levels similar to those in CLP+PBS animals. Survival of the glucose supplemented group was identical to that of the CLP group (data not shown), indicating that, although glibenclamide-induced hypoglycemia was contributing to the higher mortality, blockade of  $K_{ATP}$  channels did not provide any benefit in sepsis. Our data are in line with the observation that selective

$K_{ATP}$  blockade with sulphonylurea derivatives may not be helpful in the management of septic shock (Singer *et al.*, 2005). Furthermore, there is an apparent dissociation between the sulphonylurea receptor and the channel pore in prolonged sepsis *in vitro* (O'Brien *et al.*, 2005). In this way, the use of specific pore blockers of  $K_{ATP}$  channels may be more interesting than sulphonylurea derivatives.

Our study has two main limitations. The first is that our pharmacological approach relied on several compounds with limited selectivity (except 1400W), because highly selective blockers for some targets discussed here are still lacking. The second limitation is a general one concerning the translation of animal studies to human conditions. Sepsis is no exception. For example, animal sepsis models develop a rapid disease as compared with the human condition and do not fully resemble the human disease or involve care identical to that delivered to patients with sepsis (see Buras *et al.*, 2005).

We have not yet established the mechanism by which the early blockade of potassium channel reduces NOS-2 expression; this is currently being investigated in our laboratory. However, in spite of this caveat, the data presented here point out to a hitherto unexamined possibility of interfering with NO pathway in sepsis, namely the beneficial effect of a *partial*



**Figure 10**

Effect of NOS-2 inhibitors on NO<sub>x</sub> plasma levels. A. Effects of aminoguanidine (AG). B. Effect of 1400W. Rats were treated subcutaneously 12 h after CLP surgery with vehicle or inhibitors as in Figure 9. Twelve hours later, blood samples were collected and assayed for NO<sub>x</sub> levels. Each bar represents the mean of 8–10 animals, and vertical lines are SEM. Statistical analyses were performed using two-way ANOVA followed by Newman–Keuls *post hoc* test. \**P* < 0.05 significantly different from corresponding sham group and #*P* < 0.05 significantly different from corresponding CLP rats.

reduction in NOS-2 expression, as distinct from *total* inhibition. The results presented here suggest that blocking potassium channels with TEA early after the onset of sepsis has several beneficial consequences, such as reduction of inflammatory response and improvement in cardiac and vascular conditions, leading to better organ perfusion. Probably due to the overall improvement in the condition of the animals, survival substantially increased. The beneficial haemodynamic effects caused by TEA in rats with septic shock seem to be, at least in part, due to a partial reduction in NOS-2 expression. Because selective blockade of voltage-gated and ATP-sensitive potassium channels have not reproduced the beneficial effects of TEA, the calcium-activated potassium channels are the more likely sites of action of TEA.

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## Conflict of interest

The authors declare no conflict of interest.

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